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Update on the Prevalence of Serum Antibodies (IgG and IgM) to Adeno-Associated Virus (AAV)

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In view of presumed non-pathogenicity, tumor suppressive properties, and site-specific integration of the viral genome the human parvovirus, adeno-associated virus (AAV) has gained great interest as a gene transduction vector. Data on the seroprevalence of antibodies to AAV vary between reports, probably due to the different serological methods used. In order to understand better the immune response to AAV during natural infection, sera from different age groups and various geographical regions were compared for AAV antibodies using an ELISA. The data show that the prevalence of antibodies to AAV is similar in Europe (Germany, France, and Switzerland), Brazil, and Japan, indicating worldwide infection. It was confirmed that infection takes place during childhood. However, declining seropositivity thereafter and a second increase of seropositivity after 30 years of age suggests reinfection or reactivation of latent virus in particular as the prevalence of IgM antibodies in adults is relatively high. Furthermore, pregnant women were found to be significantly more frequently seropositive than non-pregnant controls, hinting at a reactivation of persistent AAV (up to 80% of women carry AAV in genital tissue) in specific hormonal conditions, e.g., pregnancy. Cross-reaction of serum antibodies with the different AAV types (defined by complement fixation) was observed by ELISA and neutralization tests confirming earlier results. The results suggest an unstable AAV antibody response allowing lifelong reinfection or reactivation of persisting virus possibly due to partial immunotolerance after an infection in utero, at delivery or during early infancy. ***J. Med. Virol. 59:406–411, 1999.***

KEY WORDS: parvovirus; serology; ELISA

INTRODUCTION

Adeno-associated viruses (AAV) are helper-dependent human parvoviruses usually requiring the functions of adenovirus, herpes viruses, vacciniavirus or human papillomaviruses [Berns et al., 1987; Walz et al., 1997], or genotoxic stress for replication [for reviews, see Berns et al., 1995; Schlehofer, 1994]. Without helper virus, AAV DNA can be integrated into the host cell genome at least in cell culture [Kotin et al., 1990; Samulski, 1993; Walz and Schlehofer, 1992]. By PCR, AAV was detected in human genital tissue [Friedman Einat et al., 1997; Han et al., 1996; Tobiasch et al., 1994]. AAV has been found in human abortion material [Tobiasch et al., 1994] and amnion fluids [Burguete et al., 1998]. The virus has not been associated with any disease. In contrast, AAV has tumor suppressive properties [for review, see Schlehofer, 1994]. Since, in addition, AAV DNA integration seems to occur at a specific chromosomal site [Kotin et al., 1990], the virus has been investigated for its use as a vector for gene therapy [Hallek et al., 1996]. Using complement fixation, four serotypes of AAV have been described [Hoggan et al., 1966]; in 1984, AAV-5 was isolated and characterized [Bantel Schaal et al., 1984, 1999; George Fries et al., 1984; Chiuorini et al., 1999]. Recently, an isolate from adenovirus stocks has been proposed to be of type 6 due to sequence variation and weak cross-reaction with other types, however closely related to type 1 [Rutledge et al., 1998].

Antibodies to AAV in humans were first described by Blacklow et al. [1968], using a neutralization test. It was shown that in many cases AAV infection occurs in childhood and that the prevalence of antibodies reaches a maximum (70%) in the age group of 5–10 years. Only 50% of adults (aged 18 to 30 years) were found to be seropositive. Subsequent seroepidemiologic studies have shown a reduced frequency of antibodies to AAV in tumor patients, especially in the case of genital tumors [Sprecher Goldberger et al., 1971; Mayor et al., 1976; Georg Fries et al., 1984].

However, the data reported for seroprevalence of AAV antibodies in adults vary between 35% positive

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[Sprecher Goldberger et al., 1971] and 70 to 80% [Mayor et al., 1976; Tobiasch et al., 1994] probably due to the varying serologic methods (complement fixation, immunofluorescence, and ELISA). In view of these discrepancies, a large number of samples from different age groups and several geographical regions were tested using a recently developed ELISA [Tobiasch et al., 1994].

Since the detection of AAV DNA in abortion material and amnion fluid may indicate a possible reactivation of latent AAV due to hormonal influences during pregnancy, sera from pregnant women were also tested for the presence of AAV antibodies. In order to determine whether the AAV antibodies detected by ELISA were able to neutralize, some of the sera were examined in a neutralization test for AAV-2. Assays for neutralizing antibodies to AAV types 3 and 5 were included to assess the type-specificity of possible neutralization. In addition to confirming the prevalence of AAV antibodies in childhood and adolescence, evidence on reinfection or reactivation of latent virus after the age of 30 and during pregnancy is presented. This is supported by the relatively frequent detection of IgM antibodies to AAV despite a high seroprevalence of IgG.

MATERIALS AND METHODS

Preparation of AAV Antigen for ELISA

Adeno-associated virus type 2 (AAV-2) was propagated in HeLa cells and purified using a $CsCl_2$ gradient as described previously [de la Maza et al., 1980]. AAV-2 empty capsids, kindly provided by the group of Dr. J. Kleinschmidt, were prepared using a recombinant adenovirus (courtesy of Dr. R. J. Samulski) and purified as described previously [Wistuba et al., 1997].

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was carried out using a modification of the protocol described previously . Briefly, 96-well microtiter plates (Poly-Sorp, Nunc, Wiesbaden, Germany) were coated overnight at 4°C with purified AAV-2 virions or recombinant AAV particles (50 μl/well) diluted in PBS. After washing the plates three times with PBS/0.05% Tween 20 (Sigma, Deisenhofen, Germany) the plates were blocked with Blocking Reagent for ELISA (Boehringer-Mannheim, Mannheim, Germany) for 1 hour at room temperature. After removal of the blocking buffer, the wells were incubated with serum samples diluted 1:50 to 1:400 in blocking buffer for 1 hour at room temperature. This was followed by three washing steps with PBS/Tween and 1 hour incubation with peroxidase-labeled goat anti-human IgG antibody (Dianova, Hamburg, Germany) diluted 1:2,000 in PBS/Tween. After three washing steps (PBS/Tween 0.05%) the plates were incubated with the substrate solution, [0.4 mg OPD (Sigma, Deisenhofen, Germany)/0.4 μl hydrogen peroxide 30% per ml in phosphate- citrate buffer (0.05M Na_2HPO_4, 0.025 M citric acid)]. The reaction was stopped after 10 minutes by adding 1N H_2SO_4. OD values were determined at a wavelength of 492 nm using an ELISA photometer (MWG Biotech, Ebersberg, Germany). The OD value of the background signal (serum replaced by blocking buffer) was subtracted from the sample values. Samples were considered positive when the OD was at least 3-fold higher than the negative control (AAV-2- antibody-negative human serum confirmed by immunofluorescence; cf. below).

For specific detection of IgM antibodies, sera were treated with FREKA-Fluor (Fresenius, Bad Homburg, Germany) to absorb IgG-antibodies prior to IgM-ELISA and revealed using a peroxidase labeled goat-anti human IgM antibody (Dianova, Hamburg, Germany).

Positive and negative control sera were chosen from serum samples that had previously been tested for AAV-2 antibodies using an immune fluorescence assay (see below).

Control sera were tested with both antigen coatings, full infectious particles, and empty capsids, and produced the same results. The test could be carried out with serum or plasma samples.

Immunofluorescence

The plasmid pCMV-VP expressing the AAV capsid proteins VP 1, 2, and 3 was used to transfect 293-T or HeLa cells [Chen et al., 1987] . Plasmid DNA (15 μg/10 cm dish) was incubated with 125 mM $CaCl_2$ + BBS [25 mM BES, 140 mM NaCl, 0.375 mM Na_2HPO_4, and 0.375 mM NaH_2PO_4 (pH 6.95)] for 30 minutes at room temperature before adding to the cell culture medium. Cells were then incubated at 35°C, 3% CO_2 for 16 hours. After transfection cells were seeded on 10-well multitest slides (Roth, Karlsruhe, Germany) at a density of 3×10^3/well, grown over night at 37°C, 5% CO_2, and fixed in methanol (10 minutes at −20 °C) and then in acetone (10 minutes at −20°C). After air-drying the slides were stored at −20°C.

Serum samples were diluted 1:10 in PBS, pH 7.5, and incubated on the cells for 1 hour at room temperature. The slides were washed three times with PBS and then incubated with FITC- labeled goat anti human IgG antibody (Dianova, Hamburg, Germany) diluted 1:200 in PBS for 1 hour at room temperature. After three times washing in PBS the slides were mounted with Permafluor (Coulter Immunotech, Hamburg, Germany).

Sera were considered positive if the cells showed a bright fluorescent signal in the nucleus. For detection of IgM antibodies the sera were preincubated with FrekaFluor (Fresenius, Bad Homburg, Germany) and a FITC-labeled goat anti human IgM antibody (Dianova, Hamburg, Germany) was used as second antibody.

Neutralization Test

Serum samples were diluted 1:10 and 1:50 in 50 μl of DMEM (Sigma, Deisenhofen, Germany) and incubated with virions of the respective AAV type (AAV-2, AAV-3 or AAV-5, 10^6 Infectious Particles /ml) for 3 hours at room temperature.

HeLa cells were seeded on 6-well multitest slides

(Medco, München, Germany) and infected with adenovirus type 2 as a helper, three hours prior to infection with AAV. Cells were then incubated with the serum/AAV mixture for 16 hours at 37°C, 5% CO_2, and thereafter fixed in methanol (10 minutes at –20°C) and then in acetone (10 minutes at –20°C).

Replication of AAV-2 or AAV-3 was detected using the monoclonal anti-rep antibody 76.3 [Wistuba et al., 1995]; for AAV-5 the monoclonal antibody A5 was used [Georg Fries et al., 1994]. Cells on slides were incubated with the respective antibody for one hour at room temperature followed by three washing steps in PBS and an incubation with a FITC-labeled goat anti -mouse antibody (Dianova, Hamburg, Germany) for 1 hour at room temperature. Sera were considered positive for neutralizing antibodies if no AAV specific nuclear fluorescence could be detected.

Serum Samples

A collection of 124 sera from Germany, taken for routine diagnostic work, was provided by the Department of Virology, University of Heidelberg; 57 samples were collected from laboratory-workers at the at the DKFZ Heidelberg; 23 samples of blood donors were obtained from the Virology Department of the Free University of Berlin; 196 sera from Brazil, taken from healthy blood-donors, were provided by Dr. E. Armbruster, Instituto Butantan, University of Sao Paulo; and 252 sera from Japan taken from students aged between 20 and 30 years were provided by Dr. S. Hino, Tottori University, Yonago, Japan.

The sera of pregnant women were obtained as follows: 135 sera were obtained from Dr. P. Bischof of the Department of Gynecology of the University of Geneva. The sera were taken for other diagnostic reasons between the 15th and 18th week of gestation. Dr. I. Jochmus (DKFZ Heidelberg) provided 38 sera of pregnant women from Germany. Of these, 5 women were in the first trimester, 3 in the second and 28 in the third trimester, and data were not available for 2 women.

Statistics

Differences in seroprevalence were calculated using the chi^2-test (exact) for homogeneity [Altman, 1991].

RESULTS

The seroprevalence of IgG and IgM antibodies to AAV-2 was determined using an AAV- ELISA.

Age Distribution of the Prevalence of Antibodies to AAV-2

To evaluate the age distribution of seroprevalence in Germany, sera from the Department of Virology, sera of laboratory workers and blood donors from Germany were tested for AAV-2 IgG and IgM antibodies. Figure 1a shows the distribution of AAV-2 antibodies in the different age groups.

About 30% of the children younger than 10 years were positive for anti-AAV-IgG. The antibody prevalence was then found to increase until the age of 19.

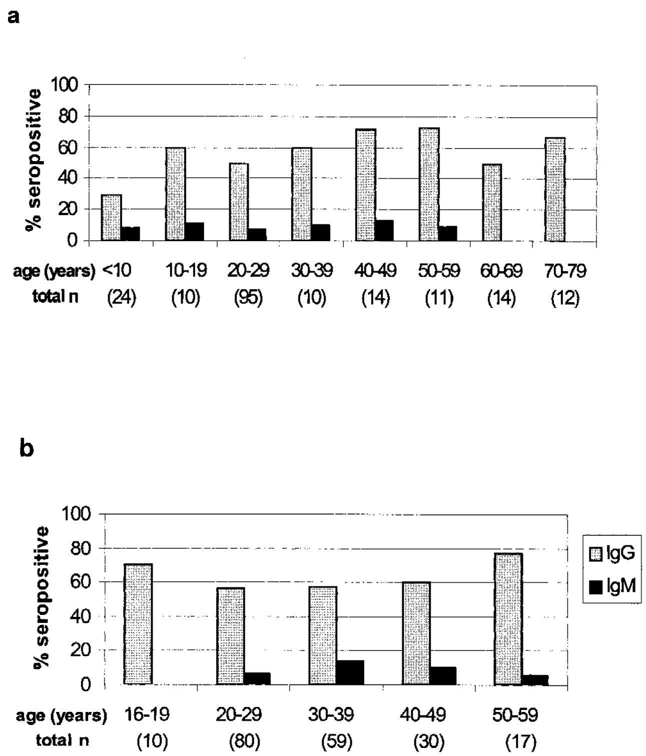

Fig. 1. Age distribution of the seroprevalence of antibodies against AAV-2 in samples from Germany (**a**) and Brazil (**b**). Gray bars represent the seroprevalence of AAV IgG antibodies; black bars represent the seroprevalence of IgM antibodies in the different age groups. Total n is the number of all samples tested for the respective age group (positive and negative sera).

The age group of 20–29 years showed a lower number of IgG positives (49% vs. 60% in the age group of 10–19 years). From the age of 30 on, the number of AAV-2 antibody positive individuals was found to be increasing, with the highest percentage in the age group 50–59 years (73%). In total, of females, 63% were seropositive for AAV-2, whereas only 41% of sera from males had AAV-2 IgG antibodies, a statistically significant difference ($P = 0.015$).

Starting with a prevalence of 6% in children under the age of 10, IgM antibodies to AAV-2 increased with age and were most frequently found in the age group of 40–49 years (13%) followed by a decrease. None of the 26 sera of individuals aged over 60 years contained IgM type antibodies to AAV-2 (Fig. 1a).

Comparison of Different Geographical Regions

To determine whether the observed age distribution only reflects AAV-infection in Germany, serum samples from other geographical regions were tested. When comparing the 196 samples from Brazil for the prevalence of AAV antibodies in the various age groups a distribution very similar to that of Germany was found (Fig. 1b).

For the age group of 20–29, it was possible to additionally analyze 252 sera from Japan. These Japanese serum samples were compared with sera from Ger-

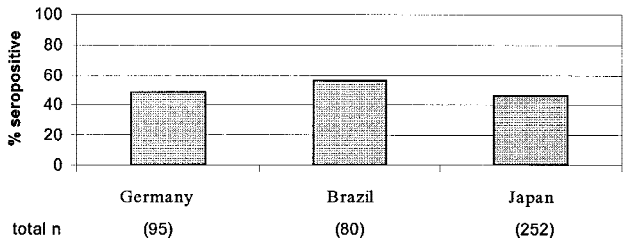

Fig. 2. Prevalence of IgG antibodies against AAV-2 in 20- to 29-year-old persons from different geographical regions. Total n is the number of all samples tested for the respective geographical region (positive and negative sera). Data for Germany and Brazil are the same as in Figure 1.

many within the same age group (20–29 years). As shown in Figure 2, 46% of the Japanese samples were AAV-2 positive compared to 49% in Germany and 56% in Brazil. The differences between the regions were not statistically significant ($P = 0.251$).

Antibodies to AAV-Helper Viruses

AAV infection is thought to occur in combination with helper virus infections, notably adenoviruses or herpesviruses. In order to determine whether the frequency of infection with these viruses was similar in Japan and Germany, the prevalence of antibodies to adenovirus, herpes simplex virus (HSV) and cytomegalovirus (CMV) in the sera from Japan and Berlin was tested in collaboration with the Institute of Virology in Berlin, Germany.

Most of the sera tested revealed antibodies to adenovirus (up to 95%). Antibodies to CMV were much more frequent in the samples from Japan (70%) compared with Germany (35%). However, AAV positive samples were just as likely to be positive for CMV antibodies or adenovirus antibodies as AAV negative samples. Antibodies to HSV were found more frequently in samples from Germany (55% compared to 38% for Japan). In Germany, the AAV positive group had a higher prevalence of HSV antibodies (64%) than the AAV negative group (42%); however this was not statistically significant ($P = 0.5$).

Higher Frequency of Antibodies to AAV-2 in Pregnant Women

Previous data indicated that hormonal influences (e.g., pregnancy) might re-activate AAV persisting in the female genital tract [Rabreau et al., 1994; Tobiasch et al., 1994]. Therefore 38 serum samples from pregnant women from Germany were tested. Control sera from 31 women of the same age group as these pregnant women (20–40 years) were chosen from the German serum collection. To the best of our knowledge these women were not pregnant. It was found that pregnant women had a much higher frequency of IgG antibodies to AAV-2 than those in the control group. A statistically significant difference ($P = 0.003$) was observed when comparing the seroprevalence of AAV-2 IgG antibodies in pregnant women (89.5%) to that of the control group (55%) (Fig. 3). A similarly high seroprevalence of 78% was found for 138 sera of pregnant Swiss women. The results of these sera were compared to those of the control sera from Germany (control sera from Switzerland were not available), which also resulted in a significant difference ($P = 0.021$).

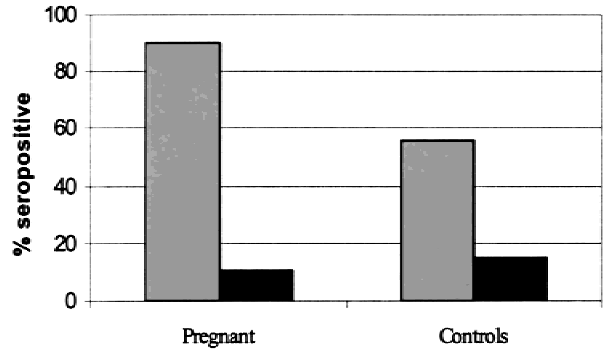

Fig. 3. Prevalence of antibodies against AAV-2 in pregnant women compared to controls. Gray bars represent the seroprevalence of AAV IgG antibodies; black bars represent the seroprevalence of IgM antibodies. Total n is the number of all samples tested (positive and negative sera). Statistics: $P = 0.003$ on the test for no association of IgG prevalence in the group of pregnant women vs. the control group.

The high prevalence of IgG antibodies was not paralleled by an increased prevalence of IgM antibodies. Pregnant women had IgM antibodies to AAV-2 at a similar frequency to control individuals [10.5% (pregnant, Germany), 11% (pregnant, Switzerland), vs. 15% (controls, Germany)].

Neutralizing Antibodies to AAV

To determine whether the antibodies detected by ELISA or immunofluorescence test have neutralizing properties, samples chosen at random were tested by a neutralization test. Forty sera tested previously by ELISA (32 sera positive for AAV-2 and 8 sera negative for AAV-2) were investigated for the presence of neutralizing antibodies to AAV-2. It was found that out of the 32 positive sera, 27 sera also had neutralizing antibodies, whereas the 8 negative sera did not neutralize AAV-2.

Of the 40 sera pre-tested by ELISA, 8 could be tested in parallel for neutralizing antibodies to three different AAV serotypes (AAV-2, -3, and -5). Six of these sera were positive for AAV-2 by ELISA and 2 were negative. Of these 6 ELISA positive sera, 3 had neutralizing antibodies to AAV- 2, 4 to AAV- 3, and 3 to AAV-5. One serum neutralized all three types (cf. Table I). From the ELISA negative sera (negative for AAV-2), one had neutralizing antibodies to AAV 3 and 5.

DISCUSSION

We demonstrated here that antibodies to AAV-2 are present in the population of various geographical regions at about the same frequency. Previous studies have shown that AAV antibodies can be detected early in childhood [Blacklow et al., 1968; Schlehofer et al., 1996]. It was found that about 50% of the sera collected

TABLE I. Prevalence of Neutralizing Antibodies Against Different AAV Serotypes*

Serum no.	ELISA AAV-2	Neutralizing antibodies against AAV-2	AAV-3	AAV-5
1	+	+	+	−
2	+	+	+	+
3	+	−	+	−
4	+	−	−	+
5	+	+	−	+
6	+	−	+	−
7	−	−	+	+
8	−	−	−	−

*Eight sera tested by ELISA (coated with AAV-2 capsids) were additionally tested using a neutralization test for AAV-2, AAV-3 and AAV-5.

from the group aged 20–29 years were anti-AAV-IgG positive and that the number of IgG positives then increased up until the age of 50–59 years. A similar increase was found for IgM antibodies reaching its maximum in the age of 40–49 years. IgM positive individuals also showed IgG antibodies in most cases. This indicates that reinfections might occur or that AAV is re-activated from a latent state. Reactivation of AAV could be due to infections together with helper viruses, e.g., HSV or HPV. Data on the prevalence of IgG antibodies to some helper-viruses (HSV, CMV, and Adenovirus) were only available for the sera from Japan and Berlin (20- to 30-year-old persons) and in these study groups there was no association found between the frequency of serum antibodies to AAV and one of its helper viruses. So far the data do not indicate that reactivation of AAV requires adenovirus infection since other helper viruses could also play a role. In the genital tract AAV replication seems to be associated with HPV [Walz et al., 1997] or CMV infection (unpublished results).

On the other hand, reactivation could occur as a consequence of hormonal influences that activated helper-viruses. For example, it is known that CMV is activated during pregnancy [Schmitz et al., 1977] and this was also considered for HPV [Chang-Claude et al., 1996; Smith et al., 1991]. This would agree with the finding that the prevalence of AAV IgG antibodies is much higher in pregnant women compared to women of the same age group. Most sera from Germany (28 out of 38) were taken at week 25 to 36 of pregnancy, those from Geneva at week 15 to 18. There were no age-matched control samples of non-pregnant women available. Therefore the data of pregnant women were compared to those of women of the same age group (20 to 40 years). It cannot be excluded that some of these women in the control group were pregnant (information was not available). Therefore the percentage of positives might be overestimated in these controls and hence the difference could be even greater when using a better defined control, i.e., non-pregnant women. Perhaps pregnant women in the population examined contribute to the higher seroprevalence in women compared to men. It was surprising that IgG antibodies to AAV were found more frequently during pregnancy with no elevated IgM prevalence. IgM antibodies may only be present for a short period; thus sera should be taken early in pregnancy to detect IgM. In contrast to these findings with normal pregnancy, in women with early abortions, a higher frequency of IgG antibodies was not found in a previous study [Tobiasch et al., 1994]. However women with early miscarriage were more often positive for IgM antibodies. Reactivation or reinfection during pregnancy might lead to infection of the embryo and might result in partial immunotolerance to AAV, explaining the instability and apparently non-protective effect of antibodies to reinfection, infection with different types of AAV, or reactivation of latent AAV later in life.

It was possible to detect neutralizing antibodies to AAV-2 in most of the sera found to be positive by ELISA confirming the specificity of the results obtained by ELISA. Some patients also had neutralizing antibodies to AAV-3 and -5 indicating that cross-neutralization exists but is not complete since sera positive for only one serotype of AAV were also found Alternatively, these findings may indicate that persons with antibodies against more than one type of AAV had infections with several AAV types at different times. However previous data obtained by PCR indicate that AAV-2 is the most frequent type present in human material, AAV-3 and AAV-5 being rather rarely detected [Tobiasch et al., 1998].

In summary, it was shown that the immune response to AAV infection may not protect from reinfection or reactivation. More detailed studies will be needed to assess the role of the presence of (cross-) neutralizing antibodies in humans and the possible instability of immunity notably with regard to the use of this virus as a tool for gene therapy.

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